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Triamcinolone acetonide alleviates benign biliary stricture by ameliorating biliary fibrosis and inflammation

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We conducted a comprehensive series of molecular biological studies aimed at unraveling the intricate mechanisms underlying the anti-fibrotic effects of triamcinolone acetonide (TA) when used in conjunction with fully covered self-expandable metal stents (FCSEMS) for the management of benign biliary strictures (BBS). To decipher the molecular mechanisms responsible for the anti-fibrotic effects of corticosteroids on gallbladder mucosa, we conducted a comprehensive analysis. This analysis included various methodologies such as immunohistochemistry, ELISA, real-time PCR, and transcriptome analysis, enabling us to examine alterations in factors related to fibrosis and inflammation at both the protein and RNA levels. Overall, our findings revealed a dose-dependent decrease in fibrosis-related signaling with higher TA concentrations. The 15 mg of steroid treatment (1X) exhibited anti-fibrosis and anti-inflammatory effects after 4 weeks, whereas the 30 mg of steroid treatment (2X) rapidly reduced fibrosis and inflammation within 2 weeks in BBS. Transcriptomic analysis results consistently demonstrated significant downregulation of fibrosis- and inflammation-related pathways and genes in steroid-treated fibroblasts. Use of corticosteroids, specifically TA, together with FCSEMS was effective for the treatment of BBS, ameliorating fibrosis and inflammation. Our molecular biological analysis supports the potential development of steroid-eluted FCSEMS as a therapeutic option for BBS in humans resulting from various surgical procedures. [BMB Reports 2024; 57(4): 200-205]

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INTRODUCTION

Benign biliary stricture (BBS) results from excessive collagen deposition, due to prolonged chronic inflammation and active myofibroblast proliferation, thickening the bile duct epithelial cells (1). BBS typically arises due to factors like acute inflammation, improper clip placement, and the induction of adjacent tissue fibrosis by stent application (2). As BBS progresses, transforming growth factor beta (TGF-β) is secreted, initiating the activation of the related signaling pathways; consequently, resulting in an upregulation of genes like transforming growth factor beta receptor 1 (TGFBR1), alpha-smooth muscle actin (α-SMA), and Collagen type 1 (3, 4). Furthermore, genes associated with the SMADs signaling pathway, a downstream pathway of the TGF-β signaling pathway, such as SMAD2 and SMAD3, show an increasing tendency. When a stricture develops in the biliary tract, it triggers an inflammatory response. During chronic inflammation, pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) are known to enhance the expression of enzymes such as cyclooxygenase-2 (COX-2). Additionally, cytokines such as transforming growth factor-beta (TGF-β), which play a crucial role in promoting fibrosis, are secreted during chronic inflammation (5).

The need to treat BBS arises due to the current inadequacies in the treatment landscape and the insufficient research on this condition. Consequently, there is a pressing need for research to develop more therapeutic strategies. According to recent reports, treatment of BBS includes various approaches such as endoscopic retrograde cholangiopancreatography (ERCP), percutaneous transhepatic cholangiography (PTC), balloon dilatation, and stent replacement (6-8).

In our previous study, we assessed the therapeutic impact of a newly developed drug-eluting metal stent, incorporating corticosteroids, triamcinolone acetonide (TA), known for their anti-fibrotic and anti-inflammatory properties (9). This evaluation was conducted using a BBS pig model. The stent was implanted in the mini-pig's bile duct for one month to investigate its effects on BBS. Furthermore, we monitored the formation of

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bile sludge within the stent lumen over the same one-month period.

In this study, we aimed to elucidate the molecular biology mechanisms underlying the anti-fibrotic and anti-inflammatory effects of corticosteroids on gallbladder mucosa. To explore the mechanistic effects of steroid biliary stents in a newly established BBS pig model, we conducted various assessments with the obtained tissue, including immunohistochemical analyses, ELISA, real-time PCR analyses, and transcriptome analysis. These analyses allowed us to measure changes in factors related to inflammation and fibrosis at both the protein and RNA levels. However, many of the molecular mechanisms underlying the anti-fibrotic and anti-inflammatory signaling of steroid-eluting stents remain unclear.

While analyzing the protective effects of steroid biliary stents against fibrosis and inflammation as revealed by immunohistochemical analyses, ELISA, real-time PCR analyses, and transcriptome analyses in the BBS pig model, we observed which pathways were significantly suppressed. Our findings indicate that concurrent treatment with steroids effectively inhibits the fibrotic response and inflammatory reaction. Consequently, targeting the genes identified as playing a significant role in our data, in combination with steroid treatment, could be an effective therapeutic strategy for BBS.

RESULTS

TA inhibits bile duct fibrosis in the BBS pig model

To investigate the anti-fibrotic effects of TA, tissue from the mini-pig biliary model was harvested for immunohistochemical staining of well-known fibrosis markers, TGF-β, TGFBR1, and α-SMA. The staining results showed a significant decrease after 4 weeks of 1X TA treatment, while samples treated with 2X TA exhibited an effective decrease starting at 2 weeks of treatment (Fig. 1A-C). In samples treated with 2X TA, the mRNA levels of genes associated with the TGF-β signaling pathway, such as TGFB1, TGFBR1, α-SMA, and COL1A1 reduced after 2 weeks of treatment, while in samples treated with 1X TA, this decline became apparent after 4 weeks of treatment (Fig. 1D, Supplementary Fig. 1A). The mRNA levels of the SMAD signaling pathway, which is a downstream pathway of the TGF-β signaling pathway, were consistent with the mRNA levels of genes related to the TGF-β signaling pathway. The mRNA levels of SMAD2, SMAD3, and SMAD4, following a pattern akin to that of genes associated with the TGF-β signaling pathway, reduced within two weeks of 2X TA treatment. In samples treated with 1X TA, this reduction was observed after four weeks. On the contrary, mRNA levels of SMAD7 increased owing to its inhibitory role (Fig. 1E, Supplementary Fig. 1B). In summary, our data directly demonstrated a significant decrease in fibrosis-related signaling with increasing TA concentration, indicating a dose-dependent increase in the anti-fibrotic effects of TA.

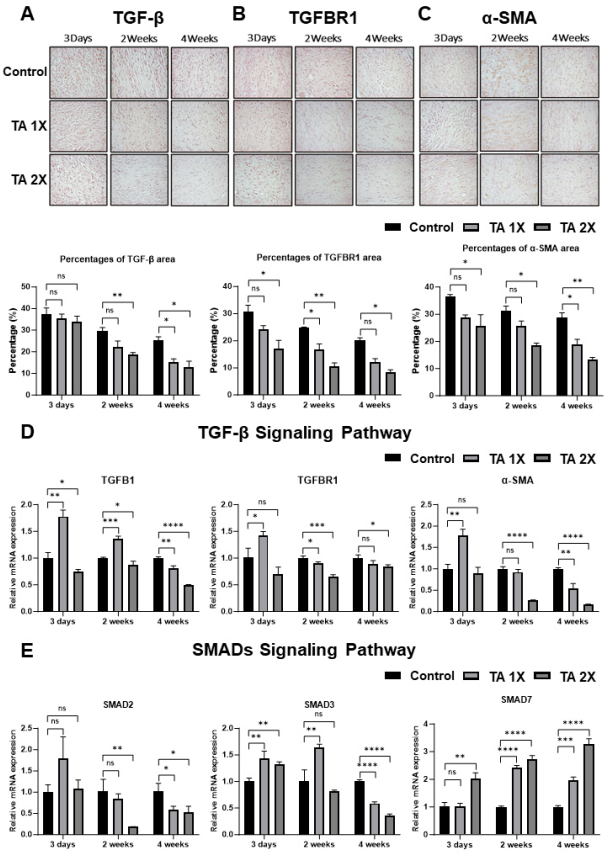

Fig. 1. TA inhibits bile duct fibrosis in the BBS pig model. Immunohistochemistry (IHC) results and percentage area of benign biliary strictures (BBS) pig model tissue treated with triamcinolone acetonide (TA) for 3 days, 2 weeks and 4 weeks, stained with fibrosis-related genes. (A) Transforming growth factor beta (TGF-β), (B) transforming growth factor beta receptor 1 (TGFBR1), and (C) alpha-smooth muscle actin (α-SMA). (D) TGF-β signaling pathway genes *TGFB1*, *TGFBR1*, α-*SMA*, and (E) SMADs signaling pathway genes *SMAD2*, *SMAD3*, and *SMAD7* mRNA expressions were assessed by real-time RT-PCR. *P $<$ 0.05, **P $<$ 0.01, ***P $<$ 0.001, ****P $<$ 0.0001, ns, not significant. Data are expressed as mean ± SEM.

TA alleviates inflammation of the bile duct in BBS pig model

To identify the anti-inflammatory effects of TA on BBS, harvested tissues were stained with inflammation-related genes, a cluster of Differentiation 68 (CD68), TNF-α, and COX-2 for immunohistochemistry (IHC). The stained samples showed a notable decrease in the percentage area with 2X TA treatment over 4 weeks and 1X TA treatment over 2 weeks (Fig. 2A-C). While mRNA levels of pro-inflammatory cytokine genes, TNF-α, IL-6, and IL-1β, were markedly decreased over time and TA concentration, anti-inflammatory cytokine IL-10 mRNA levels increased in 2 weeks of treatment (Fig. 2D, Supplementary Fig. 2A). Consistently, ELISA results confirmed the anti-inflamma-

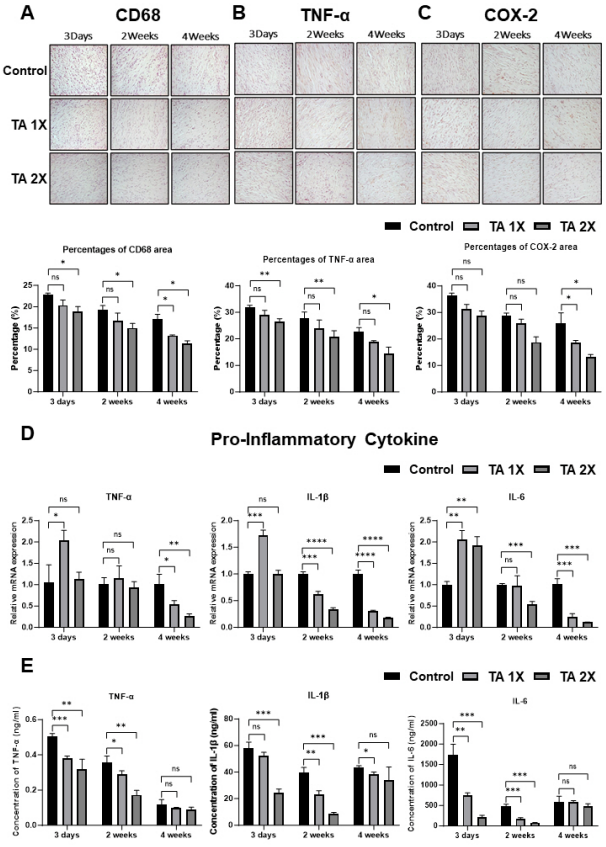

Fig. 2. TA alleviates inflammation of the bile duct in the BBS pig model. IHC results and percentage area of BBS pig model tissue treated with TA for 3 days, 2 weeks, and 4 weeks, stained with fibrosis-related genes (A) cluster of Differentiation 68 (CD68), (B) TNF-α, and (C) cyclooxygenase-2 (COX-2). (D) Pro-Inflammatory Cytokine genes *TNF-α*, *IL-6*, and *IL-1β* mRNA expressions were assessed by real-time RT-PCR. (E) Quantification of inflammatory cytokines TNF-α, IL-6, and IL-1β were assessed by ELISA assay. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns, not significant. Data are expressed as mean ± SEM.

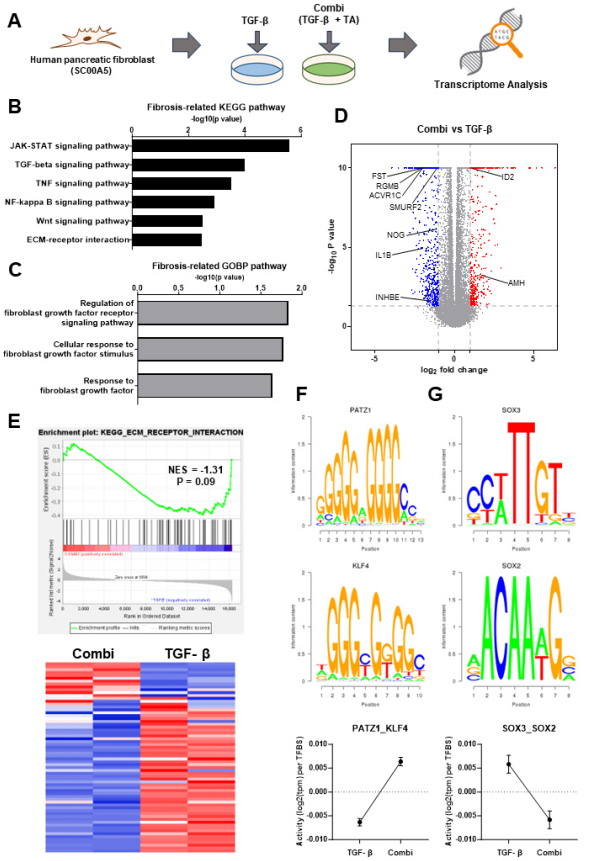

Fig. 3. Combinational treatment of TGF-β and TA increases anti-fibrotic pathways in human pancreatic fibroblasts. (A) The scheme shows transcriptomic analysis conducted with human pancreatic fibroblast (SC00A5) samples treated with TGF-β (1 ng) alone and a combination of TGF-β (1 ng) + TA (20 μM). (B, C) Significantly enriched pathways related to fibrosis. KEGG and GO databases were used for the pathway analysis. (D) Volcano plot showing significantly enriched DEGs between the two samples. (E) GSEA plot showing enriched gene sets of ECM_RECEPTOR_INTERACTIONS. Gene sets were downregulated in combinatory treated samples. (F, G) ISMARA motif analysis of key transcription factors in fibrosis. Motif activities showed anti-fibrotic effects in combinatory treated samples compared to TGF-β alone treated samples.

tory properties of TA, as evidenced by a reduction in the protein levels of pro-inflammatory genes TNF-α, IL-6, and IL-1β, along with an increase in the anti-inflammatory gene IL-10 (Fig. 2E, Supplementary Fig. 2B). Taken together, 1X TA-treated samples demonstrated anti-inflammatory effects after 4 weeks of treatment, while 2X TA-treated samples exhibited significant anti-inflammatory effects starting from 2 weeks of treatment. This indicates a dose-dependent increase in the TA anti-inflammatory effects.

Combinational treatment of TGF-β and TA enhances anti-fibrotic and anti-inflammatory pathways in human pancreatic fibroblasts

Based on our prior study and *in vitro* experiments, we performed transcriptomic analysis using human pancreatic fibroblast, SC00A5, as a model for strictures. The reason for using human pancreatic fibroblasts is that strictures are commonly observed in the pancreas and by using pancreatic fibroblasts, we believed it would provide the closest mimicry of strictures at the cellular level in the pig model. The goal was to investigate signaling changes induced by TA treatment comparing samples treated with TGF-β alone in fibroblasts to those treated with a combination of TGF-β and TA in fibroblasts (Fig. 3A). Utilizing differentially expressed genes (DEGs) identified from the transcriptome analysis data, we substantiated close interac-

tions between fibrosis- and inflammation-related pathways through pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) databases in EnrichR (10-12). Significantly enriched pathways included the TGF-beta signaling pathway, JAK-STAT signaling pathway, Regulation of fibroblast growth factor receptor signaling pathway, and Response to fibroblast growth factor (Fig. 3B, C). While the red and blue sections of the volcano plot represent DEGs, genes associated with the TGF-beta signaling pathway, a key pathway in fibrosis progression, showed significant upregulation or downregulation (Fig. 3D). Furthermore, in line with our pathway analysis findings, the enrichment plot for the fibrosis-related pathway ECM_RECEPTOR_INTERACTION exhibited downregulation in the combinatory treated sample as per the results of Gene Set Enrichment Analysis (GSEA) (Fig. 3E). To identify the transcription factors acting as key regulators, motif analysis was performed through Integrated System for Motif Activity Response Analysis (ISMARA). We observed an increased motif activity of PATZ1_KLF4, a well-known factor that activates the JAK-STAT signaling pathway and is recognized for inhibiting fibrosis, in the combinatory treated samples compared to those treated with TGF-β alone. Additionally, we noted a decrease in motif activity of SOX3_SOX2, a prominent transcription factor associated with fibrosis, in the combinatory treated samples. This observation aligns with the previous analysis results, which indicated a consistent trend in the motif activity of transcription factors (Fig. 3F, G).

Through pathway analysis using the KEGG and GO databases, we also observed that crucial pathways associated with inflammation, including the MAPK signaling pathway, IL-17 signaling pathway, Inflammatory response, and Cytokine production, were significantly upregulated (Fig. 4A, B). In the GO database, all genes associated with the key inflammation pathway, Inflammatory response, were included in the list of DEGs (Fig. 4C). This indicates a significant alteration in this pathway by the combinatory treated sample. Moreover, based on the GSEA results from the HALLMARK pathways, we validated a significant reduction in inflammation-related pathways, including TNFA_SIGNALING_VIA_NFKB and INFLAMMATORY_RESPONSE (Fig. 4D, E). In addition to the motif activity changes of transcription factors, with the combinatory treated sample, we observed a decrease in the activity of the transcription factor SP1, known to be involved in inflammatory processes. Moreover, SP3, a transcription factor recognized for its anti-inflammatory effects and closely linked to transcription factor SP1 (13), exhibited an increase, in the same sample, when compared to the TGF-β-treated sample (Fig. 4F, G).

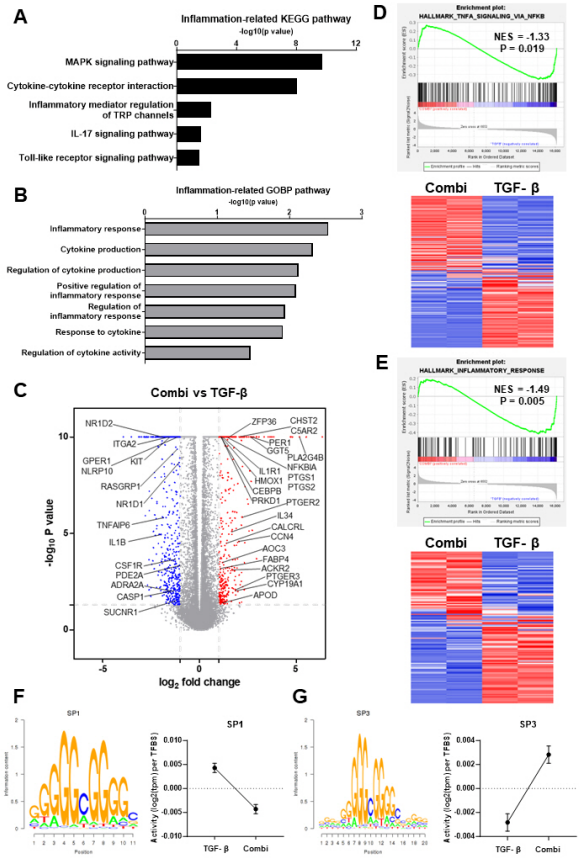

Fig. 4. Combinational treatment of TGF-β and TA escalates anti-inflammatory pathways in human pancreatic fibroblasts. (A, B) Significantly enriched pathways related to inflammation. KEGG and GO databases were used for the pathway analysis. (C) Volcano plot showing significantly enriched DEGs between the two samples. (D, E) GSEA plots showing enriched gene sets of TNFA_SIGNALING_VIA_NFKB and INFLAMMATORY_RESPONSE. Gene sets were downregulated in combinatory treated samples. (F, G) ISMARA motif analysis of key transcription factors in inflammation. Motif activities showed anti-inflammatory tendencies in combinatory treated samples compared to TGF-β alone treated samples.

DISCUSSION

BBS is often caused by surgical factors, inflammation, and anatomical irregularities, leading to excessive collagen deposition and changes in inflammation-related genes. In this study, we implanted stents for one month and monitored their impact on BBS (14). Our primary goal was to uncover the molecular mechanisms responsible for the anti-fibrotic and anti-inflammatory effects of corticosteroids on gallbladder mucosa. To explore these mechanisms of corticosteroid-eluting stents in a BBS pig model (9), we conducted a comprehensive analysis, including IHC, ELISA, real-time PCR, and transcriptome analysis, to assess alterations in factors related to fibrosis and inflammation, at both the protein and RNA levels. Our data directly showed that as the concentration of TA increased, there was a significant reduction in fibrosis-related signaling, highlighting a dose-dependent increase in the anti-fibrotic effects of TA. Additionally, 1X TA-treated samples demonstrated these anti-inflammatory effects after 4 weeks of treatment, while the 2X

TA-treated samples showed a notable increase in anti-inflammatory effects starting as early as 2 weeks of the treatment. This observation points to a dose-dependent increase in the TA anti-inflammatory effects as well. The results of the transcriptome analysis did not align perfectly with the *in vitro* data, but they exhibited a similar pattern. The reason for the lack of complete consistency can be attributed to the difference in species used for the *in vitro* experiments and transcriptome analysis. Pathways and genes associated with fibrosis and inflammation signaling exhibited significant downregulation in human pancreatic fibroblasts treated with TGF-β and TA. Similarly, transcription factors linked to fibrosis and inflammation consistently displayed a decrease in samples treated with TGF-β and TA. According to other existing studies, transcription factors SP1 and SP3 are known to have anti-inflammatory effects, which is consistent with our data.

Steroids are widely recognized for their effectiveness in treating inflammation in various tissues, including fibroblasts (15-18). Glucocorticoids, a class of steroids, are known for their potent anti-fibrotic, anti-inflammatory, and immunomodulatory properties, making them a common choice for treating a wide range of acute and chronic inflammatory conditions (19-22). However, it's worth noting that while corticosteroids are highly effective in managing inflammatory conditions like asthma, their efficacy is relatively lower in diseases such as chronic obstructive pulmonary disease (23). Corticosteroids are also beneficial in conditions characterized by swelling and irritation, such as rheumatoid arthritis and autoimmune disorders like lupus and sclerosis (24-27). In addition, recent studies have also highlighted the effectiveness of corticosteroids in treating diseases involving the suppression of fibrosis (26, 28). The most commonly used corticosteroids include TA. TA can be effective in relieving inflammation, pain, and discomfort associated with various diseases (29, 30). However, their use may lead to a range of side effects, including increased susceptibility to infections, as these drugs suppress the immune system (31). Consequently, recent studies have explored various approaches for reducing the side effects, such as adjusting medication doses, using alternative medications, or combination therapies (32, 33).

Due to the limited research conducted on the use of corticosteroids in BBS, the results and treatment outcomes of BBS are currently scarce. However, our results, along with findings from previous studies, have shed light on the effects of TA, a corticosteroid. While the identified pathways and target genes in this study show promising effects when inhibited, further research on these targets is needed. Subsequent studies on the potential therapeutic agents derived from these targets could contribute to the development of effective treatments. Moreover, if any of the target genes are amenable to drug development, using them in combination with TA may enhance therapeutic efficacy.

MATERIALS AND METHODS

Materials and methods are available in the Supplemental information.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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